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PRINCIPAL INVESTIGATOR: Blaine A. Christiansen

CONTRACTING ORGANIZATION: University of California
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| 14. ABSTRACT Current osteoporosis therapies are able to treat the symptoms of osteoporosis, however little progress has been made toward understanding and addressing the underlying mechanisms contributing to age-related bone loss, or the ability to adapt to mechanical loading (exercise). Degeneration in peripheral nerve function with age may be one of these mechanisms, as neuropeptides affect the function of osteoblasts and osteoclasts in vitro, and nerve deactivation causes bone loss in vivo. This research investigates mechanisms by which peripheral sensory nerves influence bone maintenance and mechanotransduction using capsaicin-injected mice as a model of decreased peripheral sensory nerve function. We hypothesize that decreased sensory nerve function will result in increased functional adaptation of bone. In Aim 1 we investigated the relationship between peripheral sensory nerve function and bone structure. We found that capsaicin treatment resulted in a small but statistically significant decrease in trabecular and cortical bone structure. In Aim 2 we will determine the bone adaptation response of capsaicin- and vehicle-treated mice to increased mechanical loading. We hypothesize that capsaicin-treated mice will have an increased bone adaptation response to mechanical loading. The proposed research will establish the role of peripheral sensory nerves in age-related decreases in bone's ability to adapt to exercise. These studies may lead to novel therapies aimed at preserving healthy bone turnover with age. This research will be the basis for future studies investigating the interaction of peripheral nerves and bone, and peripheral nerve function as a potential mechanism of age-related bone loss. | | | | | |
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INTRODUCTION: Osteoporosis is a major public health concern for an ever-growing aging population, affecting over 44 million Americans [1]. Osteoporotic fractures are associated with morbidity, increased mortality, and a general decrease in quality of life. While current pharmacological therapies are useful for treating symptoms of osteoporosis, little progress has been made toward understanding and addressing the *underlying mechanisms leading to age-related bone loss*. Diminished innervation of bone with age may be one of these mechanisms, as peripheral nerve function has been shown to affect bone metabolism both *in vitro* [2-7] and *in vivo* [8, 9]. The proposed research investigates the role of peripheral sensory nerve function on bone maintenance and mechanotransduction using capsaicin-treated mice as a model of decreased peripheral nerve function. We hypothesized that decreased peripheral nerve function will result in an *increased* functional adaptation response of bone, due to decreased negative feedback on osteoblast and osteoclast function. To test this hypothesis, we proposed two specific aims. The first aim determined the effects of neonatal capsaicin treatment on bone structure and metabolism compared to vehicle-injected mice. The second aim determined the bone adaptation and peripheral nerve response of capsaicin- and vehicle-injected mice to increased mechanical loading. This research will establish the role of peripheral nerves in bone metabolism and mechanotransduction, and may lead to novel therapies aimed at preserving healthy bone turnover with age.

BODY:

Approved Statement of Work (updated 5 September, 2014):

Aim 1. *Determine the effects of neonatal capsaicin treatment on bone structure and metabolism, and neuropeptide concentrations in bone compared to vehicle-injected mice (months 1-12):*

- 1a. Institutional approval of animal use protocols (*this will be done before the funding period begins)
- 1b. Capsaicin or vehicle treatment of neonatal mice (months 1-3)
- 1c. Micro-computed tomography of mouse bones (months 2-6)
- 1d. ELISA analysis of neuropeptides in bone (months 2-10)
- 1e. Multiplex analysis of bone biomarkers (months 2-10)
- 1f. Embedding/cutting/imaging/analysis of bones for dynamic histomorphometry (months 4-12)

Aim 2. *Determine the bone adaptation and peripheral nerve response of capsaicin- and vehicle-injected mice to increased mechanical loading (months 6-18):*

- 2a. Strain gage analysis of bone strain during tibial compression (months 6-7)
- 2b. Capsaicin or vehicle treatment of neonatal mice (months 6-8)
- 2c. Tibial compression of capsaicin- and vehicle-injected mice (months 8-10)
- 2d. Micro-computed tomography of mouse bones

~~2e. ELISA analysis of neuropeptides in bone ***~~

~~2f. Multiplex analysis of bone biomarkers ***~~

2g. Embedding/cutting/imaging/analysis of bones for dynamic histomorphometry

2h. ELISA analysis of bone formation and resorption biomarkers

2i. Histological analysis of neuropeptides in bone by immunohistochemistry

As of the date of this report (29 December, 2014), all items for Aim 1 have been accomplished, and this work has been published in the Journal of Musculoskeletal and Neuronal Interaction (attached). During the past 14 months we have also accomplished a majority of the tasks for Aim 2 (described below), and a manuscript describing this work is in preparation. We are currently finishing the remaining tasks for Aim 2, and will be completely finished with this work by the end of March, 2015.

Strain gage analysis of bone strain during tibial compression (2a):

Strain gage analysis was used to determine the bone strain engendered during tibial compression loading. This is necessary for mechanical loading studies, since bone strain magnitudes must be consistent between experimental groups in order to accurately compare the bone adaptation response. A total of 14 mice were used for these experiments (3 Vehicle-Male, 4 Vehicle-Female, 4 Capsaicin-Male, 3 Capsaicin-Female), and all procedures were performed post mortem. The anterior-medial aspect of the right tibia of each mouse was exposed post-mortem, and a single-element strain gage (UFLK-1-11-1L, 120Ω, TML Gages, Japan) was affixed to the bone using cyanoacrylate, centered on the bone 10 mm below the tibial plateau. Lower legs were

then loaded with tibial compression at 1 mm/second loading rate to peak compressive forces of 2-10 N, and average strain at each loading magnitude was determined for each experimental group.

Tibial compression of mice resulted in a dose-dependent increase in bone strain on the anterior-medial surface of the tibia with increasing compression force (Fig. 1). Strain magnitudes were comparable to those reported in other studies [10, 11]. Although some differences in mean were observed between groups, there were no statistically significant differences between groups for loading magnitudes of 2-8 N, and there was a large amount of within-group variance in mean strain magnitude. Therefore, in vivo loading studies used identical tibial compression loading schemes for all experimental groups.

Capsaicin or vehicle treatment of neonatal mice (2b):

A total of 73 male and female C57BL/6 neonatal mice were used for Aim 2 (Harlan Laboratories, Indianapolis, IN). Neonatal capsaicin treatment was performed as previously described [12]. Briefly, neonatal mice were given subcutaneous injections of capsaicin (50 mg/kg) or vehicle (10% ethanol, 10% Tween 80 in isotonic saline) on day 2 and 5 after birth (n = 39 vehicle, 34 capsaicin). Following capsaicin or vehicle treatment, neonatal mice were returned to normal cage activity until 12 weeks of age. Similar to what we observed for Aim 1, capsaicin treatment did not have a significant effect on the body weights of mice at 12 weeks of age (data not shown).

Hot plate analgesia meter testing of mice to determine sensory threshold:

Capsaicin- and vehicle-treated mice were subjected to hot-plate analgesia testing at 4, 8, and 12 weeks of age to determine response time to a constant thermal stimulus of 55 °C as previously described [13]. Mice were placed on a hot-plate (LE 7406, Coulbourn Instruments, Whitehall, PA) and removed after indication of discomfort, determined as twitching or licking of a hind limb or jumping, or after a maximum of 30 seconds, and the latency time of the response was recorded. The experiment was performed twice at each time point and the latency times averaged for each mouse.

Mice treated with capsaicin had significantly longer latency times when exposed to the constant thermal stimulus than vehicle-treated mice (Fig. 2). At 8 weeks of age, the average latency time of capsaicin-treated mice was 76% longer than that of vehicle-treated mice, while at 12 weeks of age the latency time was 124% longer for capsaicin-treated mice. This confirms that mice treated with capsaicin as neonates had decreased peripheral sensory nerve function, which persisted until at least 12 weeks of age.

Tibial compression of capsaicin- and vehicle-injected mice (2c):

At 12 weeks of age, all mice were subjected to tibial compression loading five days/week for two weeks. Half of the mice from each sex/treatment group were subjected to

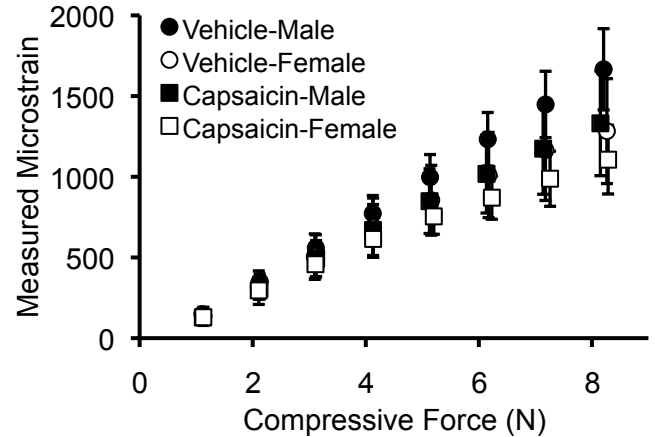


Figure 1: Measured tensile bone strain at the anterior-medial surface of the tibia during tibial compression loading. No significant differences were observed between experimental groups, therefore the same loading protocol was used for all groups for in vivo tibial compression loading.

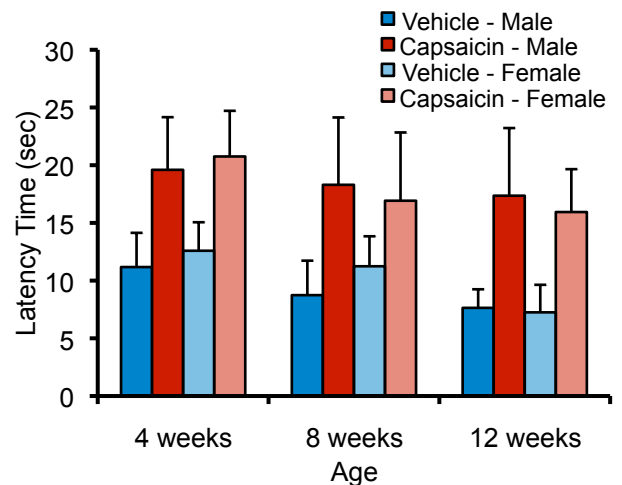


Figure 2: Latency time for hot plate analgesia testing of mice. Capsaicin-treated mice exhibited significantly longer latency time at all ages, confirming that sensory function is reduced in capsaicin-treated mice until at least 12 weeks.

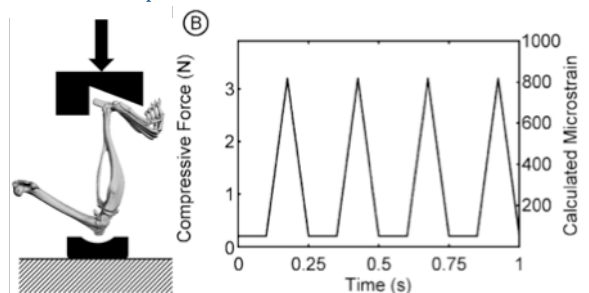


Figure 3: Tibial compression setup and example waveform. Waveform image from Fritton et al., 2005.

tibial compression loading with a target compressive load of 3 N, while the other half were loaded to a target compressive load of 7 N. Each day, mice were anesthetized using isoflurane inhalation, then the right lower leg was subjected to tibial compression loading. Axial loads were applied by an electromagnetic materials testing machine (Bose ElectroForce 3200, Eden Prairie, MN), with 4 loading cycles applied per second for a total of 1200 cycles per day (Fig. 3).

Micro-computed tomography analysis of bone structure (2d):

Bilateral tibias were removed post mortem and preserved in 70% ethanol. Bones were scanned using micro-computed tomography (SCANCO, μ CT 35, Bassersdorf, Switzerland); images were acquired at 6 μ m nominal voxel size (energy=55 kVp, intensity=114 μ A, integration time = 900 ms). Trabecular bone was analyzed at the tibial metaphysis using manually drawn contours inside the cortical shell on two-dimensional slices. The metaphysis was defined by a 900 μ m thick volume of interest beginning below the middle break of the growth plate. Trabecular bone volume per total volume (BV/TV), trabecular number, trabecular thickness (Tb.Th), and trabecular separation were determined using the manufacturer's 3-D analysis tools. Whole bone, including both cortical and trabecular bone, was analyzed at the proximal tibia, at 10%, 20%, 30%, 40%, and 50% of the bone length from the tibial plateau. Whole bone analysis used a 600 μ m thick volume of interest centered at each location. Bone area (B.Ar), medullary area (M.Ar), total cross-sectional area (Tt.Ar), bone mineral content (BMC), and cortical thickness (Ct.Th) were determined using the manufacturer's 3-D analysis tools.

Tibial compression at 3 N target compressive load resulted in significant increases in whole bone parameters in vehicle-treated female mice at 10% and 20% of the bone length (Fig. 4). Whole bone at more distal sites was not affected. Vehicle-treated male mice and all capsaicin mice exhibited no bone adaptation response to tibial compression. Similarly, no differences were observed in trabecular bone structure for any experimental groups. These data suggest a sex-based difference in bone adaptation to mechanical loading, as well as a difference between capsaicin- and vehicle-treated mice. Vehicle-treated female mice had a significant response to loading, while capsaicin-treated females had no response. This may suggest a decreased capacity for bone adaptation to mechanical loading in sensory-depleted mice. Alternatively, these findings may be emblematic of an increased threshold for initiation of adaptation in sensory-depleted mice.

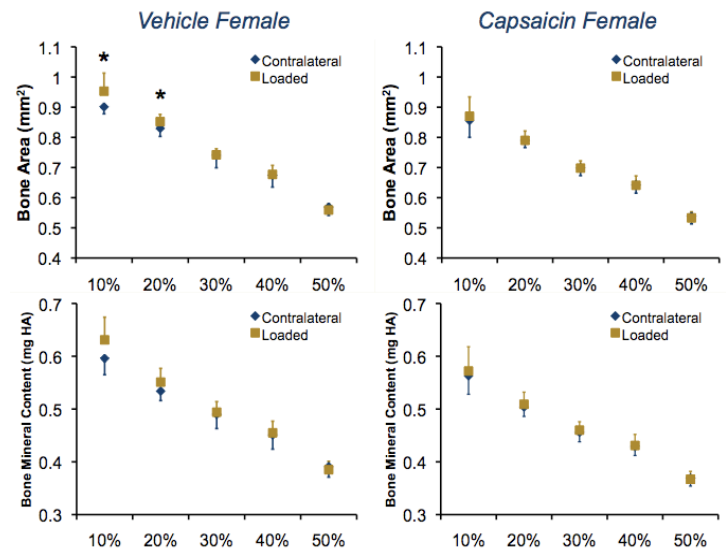
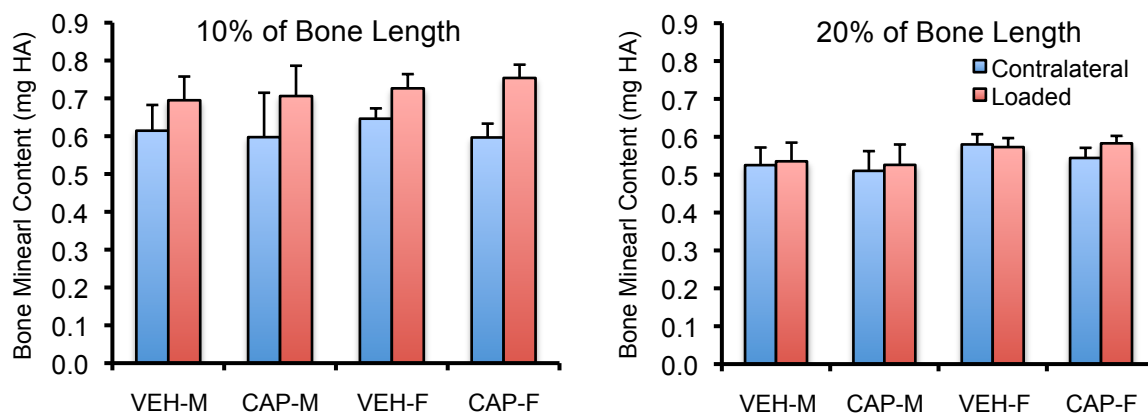


Figure 4: Whole-bone analysis of capsaicin- and vehicle-treated female mice loaded at 3N compression magnitude. Bone area was significantly increased in loaded tibiae of vehicle-treated mice, while capsaicin-treated mice exhibited no adaptation to tibial compression.

Tibial compression at 7 N target compressive load resulted in a significant bone formation response in all experimental groups, particularly at the most proximal site (10% of the bone length from the tibial plateau; Fig. 5). Bone formation at more distal skeletal sites was statistically significant, but the effect size was considerably less than at the proximal site. Whole-bone analysis indicated that capsaicin-treated mice exhibited a **greater** bone formation response than vehicle-treated mice in response to tibial compression, contrary to what was observed with 3 N loading. Trabecular bone analysis revealed a significant increase in trabecular thickness, a significant decrease in trabecular number, but no difference in BV/TV due to loading. There were no differences based on treatment (CAP or VEH) for the trabecular bone analysis.

Altogether, these data suggest that sensory-depleted mice (capsaicin-treated) may have a higher threshold for initiating an adaptation response to an anabolic mechanical stimulus, but that once this adaptation response has been activated, bone formation occurs to a greater degree than in sensory-intact (vehicle-treated) mice. Alternatively, the difference between vehicle- and capsaicin-treated mice may be distinguished by a lamellar bone formation response, which would occur at low compression magnitude, versus an injury response, which may be what is happening at the higher compression magnitude.

Figure 5: Whole-bone analysis of bone mineral content at the 10% and 20% bone length volumes of interest for 7N loaded mice. Tibial compression resulted in bone formation in all experimental groups, particularly at the 10% volume, but capsaicin-treated mice exhibited the greatest anabolic response. More distal sites also exhibited a small but significant response.



ELISA analysis of neuropeptides in bone (2e):

Femora, tibiae, muscle, and skin samples were collected from capsaicin- and vehicle-treated mice at the time of sacrifice, and were analyzed for neuropeptide concentrations using ELISA (Fig. 6). Bones were analyzed for calcitonin-gene related peptide (CGRP) and substance P (SP). Tissue samples were flash frozen with liquid nitrogen immediately following dissection, and were then crushed into small fragments, boiled in 2 M acetic acid in 4% EDTA (pH 3.5), and centrifuged at 3000 g for 15 min. Supernatants were freeze-dried and dissolved in ELISA buffer. Neuropeptide concentrations were determined using commercial mouse-specific ELISA assays (USCN Life Sciences, Inc., Wuhan, China), and data was normalized to the wet weight of the tibia to account for variation in bone size. Using this technique we were unable to detect any effects of capsaicin treatment on concentrations of CGRP or SP in bone.

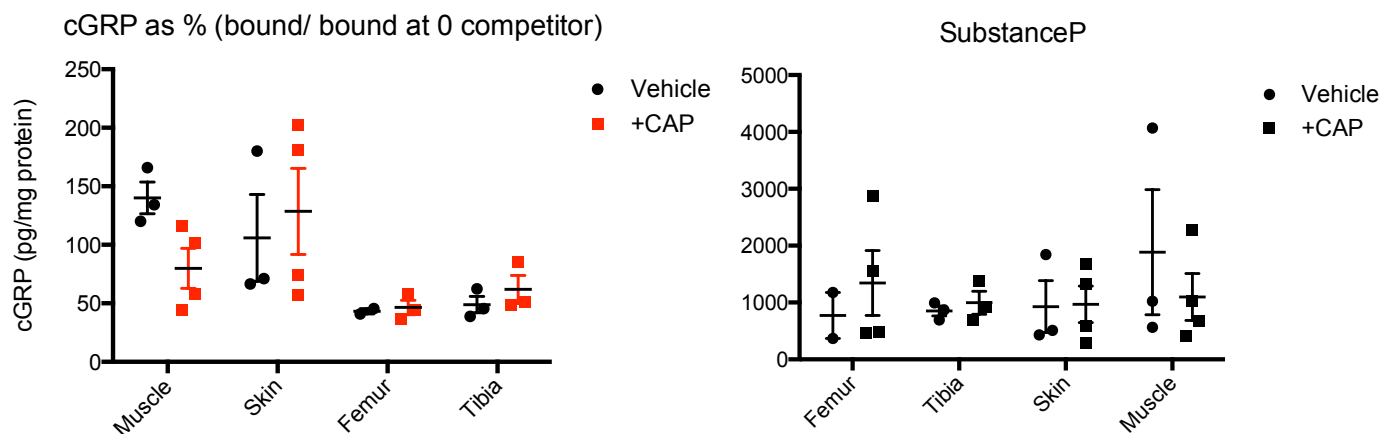


Figure 6: Concentrations of calcitonin gene-related peptide (CGRP) and substance P (SP) in bone, skin, and muscle of capsaicin- and vehicle-treated mice. No significant differences in neuropeptide concentrations were observed for any of the tissues, and a large amount of variance was present in the experimental groups. We are currently considering alternative approaches for this analysis.

Dynamic histomorphometry of fluorescent-labeled bone sections (2g):

All mice for Aim 2 received injections of calcein green (10 mg/kg; Sigma-Aldrich, St. Louis, MO) and Alizarin-3-methylimino-diacetic acid (30 mg/kg; Sigma-Aldrich, St. Louis, MO) 10 days and 3 days prior to sacrifice, respectively. After scanning with microCT, the right tibias were embedded in Technovit (Kulzer, Wehrheim, Germany) using standard techniques for undecalcified bone [14]. Sections were cut from each bone at 10%, 20%, 30%, 40%, and 50% of the bone length from the tibial plateau, using a bandsaw (Model 310, Exakt Technologies, Norderstedt, Germany) in the transverse plane. Sections were ground to an approximate thickness of 40 μ m. Two color fluorescent images were obtained at 10x magnification (Nikon Eclipse TE2000-E, Tokyo, Japan). Dynamic histomorphometric analysis are currently being performed using commercial software (Bioquant, Nashville, TN). At each bone location we will quantify mineral apposition rate (MAR), percent mineralizing surface (MS/BS), and bone formation rate (BFR) on the endosteal and periosteal surfaces. This analysis is ongoing, and has not yet produced preliminary data.

Pending tasks:

Our initial attempts at quantifying neuropeptides in bone (2e) and biomarkers of bone turnover (2f) did not yield the expected results, and it was determined that alternative methods were needed to gather this data. For Aim 1, we used ELISA analysis of bone biomarkers (replaced 1e). For Aim 2, we will again use ELISA to quantify biomarkers of bone formation and resorption (2h) and we will use advanced histological techniques to quantify neuropeptide concentrations in bone (2i).

KEY RESEARCH ACCOMPLISHMENTS:

- Conducted strain gage analysis of bone strain during tibial compression in order to normalize bone strain magnitudes for Aim 2
- Quantified adaptation of metaphyseal trabecular bone and whole bone structure in capsaicin- and vehicle-treated mice following tibial compression loading at 3N or 7N using micro-computed tomography, yielding a differential response in capsaicin-treated mice relative to vehicle-treated mice which is dependent on the compression magnitude.
- Currently conducting dynamic histomorphometry analysis for Aim 2, including mice loaded at both 3N and 7N compression magnitudes.

REPORTABLE OUTCOMES:

- Presented research in poster presentations at the 2014 International Bone and Mineral Society (IBMS) Sun Valley Workshop and the 2014 American Society for Bone and Mineral Research (ASBMR) Annual Meeting.
- Manuscript encompassing Aim 1 published in the Journal of Musculoskeletal and Neuronal Interaction.
- Manuscript encompassing Aim 2 is currently in preparation, and will be submitted to Bone by March 2015.

CONCLUSION: This research investigates mechanisms by which peripheral sensory nerves influence bone maintenance and mechanotransduction using capsaicin-injected mice as a model of decreased peripheral sensory nerve function. In **Aim 1** we quantified the relationship between reduced peripheral sensory nerve function and bone structure and mechanical properties. We hypothesized a negative correlation between reduced sensory nerve function and structure and mechanical properties of bone. Consistent with this hypothesis, we observed a small but statistically significant decrease in trabecular and cortical bone structure due to capsaicin treatment. In **Aim 2** we determined the bone adaptation response of capsaicin- and vehicle-treated mice to increased mechanical loading (tibial compression) at two different magnitudes of compression (3N and 7N). We hypothesized that capsaicin-treated mice will have an *increased bone adaptation response* to mechanical loading due to decreased negative feedback by peripheral sensory nerves. We found that tibial compression loading at 3N magnitude caused a significant bone adaptation response in female vehicle-treated mice, but no significant response in capsaicin-treated mice. In contrast to this finding, 7N tibial compression loading resulted in bone adaptation in all experimental groups, however capsaicin-treated mice exhibited the greatest adaptation response to loading. This differential dose response may be due to a higher threshold for adaptation in capsaicin-treated mice, or may be linked to a lamellar vs. injury response engendered by 3N vs. 7N loading, respectively. Altogether, this research will establish the role of peripheral nerves in bone's ability to adapt to exercise. These studies may lead to novel therapies aimed at preserving healthy bone turnover with age. This research will be the basis for future studies investigating the interaction of peripheral nerves and bone, and peripheral nerve function as a potential mechanism of age-related bone loss.

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Original Article

Altered bone development in a mouse model of peripheral sensory nerve inactivation

M.A. Heffner^{1,2}, M.J. Anderson¹, G.C. Yeh¹, D.C. Genetos³, B.A. Christiansen^{1,2}

¹University of California-Davis Medical Center, Department of Orthopaedic Surgery; ²University of California-Davis, Biomedical Engineering Graduate Group; ³University of California-Davis Veterinary School, Department of Anatomy, Physiology, & Cell Biology, USA

Abstract

Objectives: The present study sought to determine the effects of decreased peripheral sensory nerve function on skeletal development and bone metabolism in mice. **Methods:** C57BL/6 neonatal mice were treated with capsaicin to induce peripheral sensory nerve degeneration, and compared to vehicle-treated controls at 4, 8 and 12 weeks of age. Changes in bone structure were assessed using micro-computed tomography, mechanical properties and fracture resistance were assessed using three-point bending of radii, and bone turnover was assessed using dynamic histomorphometry and serum biomarkers. **Results:** Capsaicin treatment resulted in small but significant decreases in bone structure, particularly affecting trabecular bone. Capsaicin-treated mice exhibited lower trabecular thickness at the femoral metaphysis and L5 vertebral body compared with vehicle-treated mice. However, capsaicin- and vehicle-treated mice had similar mechanical properties and bone turnover rates. **Conclusion:** Neonatal capsaicin treatment affected trabecular bone during development; however these small changes may not be meaningful with respect to bone strength under normal loading conditions. It is possible that capsaicin-sensitive neurons may be more important for bone under stress conditions such as increased mechanical loading or injury. Future studies will investigate this potential role of peripheral sensory nerves in bone adaptation.

Keywords: Capsaicin, Sensory Nerves, Bone Turnover, Skeletal Development, Mechanical Testing

Introduction

Osteoporosis is characterized by decreased bone mass and increased risk of fracture¹. Common treatments for osteoporosis manipulate bone turnover primarily by inhibiting osteoclastic bone resorption^{2,3} but can also target osteoblastic bone formation⁴. However, the mechanisms underlying age-related bone loss remain elusive. Degeneration of peripheral nerves may be a contributing factor for age-related bone loss, as substantial changes in peripheral nerve structure and function occur with aging⁵⁻⁹. The reductions in axon number and fiber size in aged subjects are associated with decreased peripheral nerve function,

with cutaneous thermal and tactile thresholds significantly increased¹⁰. Further, there is now a well-established link between peripheral nerve function and bone metabolism *in vitro*¹¹⁻²¹. In addition, denervation has been shown to cause bone loss *in vivo*²²⁻²⁵, and peripheral neuropathy has been identified as an independent predictor of low bone mass in the affected limb of diabetic subjects²⁶. Despite strong evidence of a link between the function of peripheral nerves and bone metabolism, the potential role of decreased peripheral nerve function in age-related bone loss has not been investigated.

Capsaicin treatment is a useful method for isolating the effects of decreased peripheral sensory nerve function in animal models. Capsaicin treatment activates the TrpV1 receptor, which is expressed by unmyelinated and small diameter myelinated sensory neurons, but not by motor neurons, large diameter sensory neurons or sympathetic neurons^{27,28}. In this way, capsaicin treatment is able to isolate the effects of peripheral sensory neurons without disrupting normal mechanical loading, which is a confounding consequence of complete denervation. Subcutaneous injection of capsaicin in neonatal animals destroys unmyelinated and small-diameter myelinated sensory neurons for the lifetime of the animal²⁹. Neonatal cap-

The authors have no conflict of interest.

Corresponding author: Blaine A. Christiansen, Ph.D., UC Davis Medical Center, Department of Orthopaedic Surgery, 4635 2nd Ave, Suite 2000, Sacramento, CA 95817, USA
E-mail: bchristiansen@ucdavis.edu

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saicin treatment is a well-established model that has been used previously to investigate pain in rats³⁰ and itch in mice^{31,32}. We are aware of only one study that used neonatal capsaicin treatment for a study of bone metabolism in rats²². This study found that neonatal capsaicin treatment in rats did not appear to alter normal bone growth and maintenance, but that decreased sensory nerve function may decrease local bone remodeling following molar extraction. However, this study did not investigate developmental changes resulting from capsaicin treatment. Importantly, this previous study also did not quantify trabecular bone structure, and did not investigate bone mechanical properties. We are unaware of any studies that have used neonatal capsaicin treatment to investigate bone metabolism in mice.

In this study, we used neonatal capsaicin treatment in mice to investigate the effects of decreased peripheral sensory nerve function on skeletal development and bone metabolism in mice, and to establish a mouse model of decreased peripheral sensory nerve function *in vivo* for future studies in bone. Using vehicle- and capsaicin-treated mice at multiple stages of development (4, 8, and 12 weeks of age) we assessed bone structure using micro-computed tomography, mechanical properties and fracture resistance using three-point bending, and bone turnover rate using dynamic histomorphometry and serum biomarkers. We hypothesized that decreased peripheral nerve function in capsaicin-treated mice would result in decreased bone structure, decreased resistance to fracture, and decreased bone turnover rate.

Material and methods

Neonatal capsaicin treatment

A total of 42 male and female C57BL/6 neonatal mice were used in this study, from 7 timed pregnant females (Harlan Laboratories, Indianapolis, IN). Neonatal capsaicin treatment was performed as previously described³³. Briefly, neonatal mice were given subcutaneous injections of capsaicin (50 mg/kg) or vehicle (10% ethanol, 10% Tween 80 in isotonic saline) on day 2 and 5 after birth (n=21 vehicle, 21 capsaicin). Following capsaicin or vehicle treatment, neonatal mice were returned to normal cage activity until weaning (28 days). Mice were sacrificed 4, 8, or 12 weeks after birth, with a total of 7 vehicle- and 7 capsaicin-treated mice per time point (2-5 mice per age/sex/treatment group). We selected these time points to observe developmental changes from weaning until skeletal maturity, and to account for any possible recovery from sensory nerve inactivation by adulthood. Mice were weighed 1-2 times per week from birth until sacrifice.

Hot-plate analgesia testing

Capsaicin- and vehicle-treated mice were subjected to hot-plate analgesia testing at 4, 8, and 12 weeks of age to determine response time to a constant thermal stimulus of 55°C as previously described³⁴. Mice were placed on a hot-plate (LE 7406, Coulbourn Instruments, Whitehall, PA) and removed after indication of discomfort, determined as twitching or licking of

a hind limb or jumping, or after a maximum of 30 seconds, and the latency time of the response was recorded. Each mouse was tested twice at each time point, and the latency times were averaged for each mouse/time point.

Micro-computed tomography analysis of bone structure

Right tibias, right femurs, and L5 vertebrae were removed post mortem and preserved in 70% ethanol. Bones were scanned using micro-computed tomography (SCANCO, μ CT 35, Bassersdorf, Switzerland); images were acquired at 6 μ m nominal voxel size (energy=55 kVp, intensity=114 μ A, integration time=900 ms). Trabecular bone was analyzed at the metaphysis and epiphysis of the distal femur and at the L5 vertebral body using manually drawn contours inside the cortical shell on two-dimensional slices. The metaphysis was defined by a 900 μ m thick volume of interest beginning below the middle break of the growth plate. Trabecular bone volume per total volume (BV/TV), trabecular number, trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMR) were determined using the manufacturer's 3-D analysis tools. Cortical bone was analyzed at the mid-diaphysis of the tibia and femur, using a 240 μ m thick volume of interest centered at the measured midpoint of each bone. Bone area (B.Ar), medullary area (M.Ar), total cross-sectional area (Tt.Ar), cortical thickness (Ct.Th), and bone mineral density (BMR) were determined using the manufacturer's 3-D analysis tools.

Three-point bending

Bilateral radii were removed post mortem and preserved in 70% ethanol. Bones were scanned using micro-computed tomography as described above, with a volume of interest that included the entire bone. Average bending moment of inertia for the central 1.0 mm (100 slices) was determined using BoneJ analysis of the microCT images³⁵. Following μ CT scanning, radii were rehydrated for 25 minutes in phosphate buffered saline (PBS), then mechanically tested in three-point bending to determine cortical bone material properties. The lower supports had a span of 5.02 mm for 4 week samples, and a span of 7.45 mm for 8 and 12 week samples (Figure 4A), and the center loading platen was driven at 0.2 mm/sec until failure. Resulting force and displacement data were analyzed to determine stiffness and yield force. Modulus of elasticity and yield stress were determined using Euler-Bernoulli beam theory.

Dynamic histomorphometry

Mice received injections of calcein green (10 mg/kg; Sigma-Aldrich, St. Louis, MO) and Alizarin-3-methylimino-diacetic acid (30 mg/kg; Sigma-Aldrich, St. Louis, MO) 10 days and 3 days prior to sacrifice, respectively. After scanning with μ CT, the right tibias were embedded in Technovit (Kulzer, Wehrheim, Germany) using standard techniques for undecalcified bone³⁶. Two sections were cut from each bone on a bandsaw (Model 310, Exakt Technologies, Norderstedt, Germany) in the transverse plane at 40% of the length from the proximal end. Sections were ground to an approximate thickness of 40 μ m. Two color fluorescent images were obtained at 10x mag-

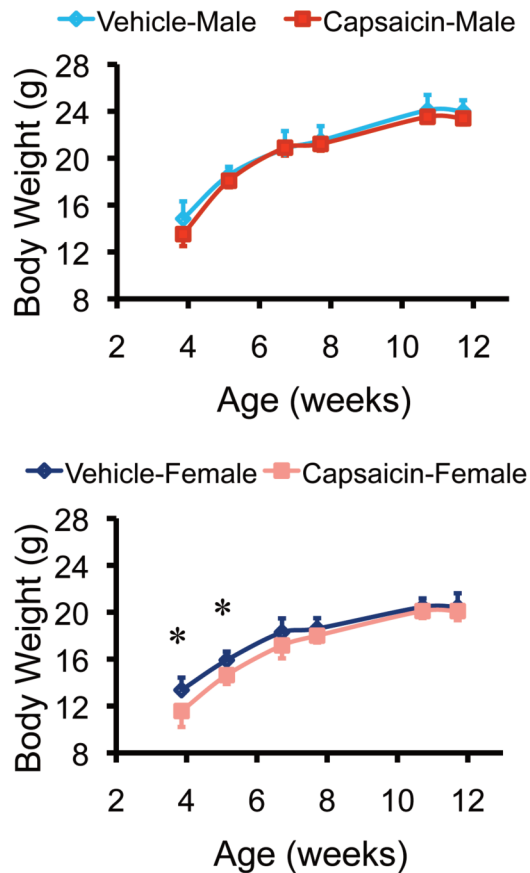


Figure 1. Body weights of treated and untreated mice were recorded from weaning until 12 weeks of age. Female mice treated with capsaicin had lower body weight than vehicle-treated female mice from weaning until day 47. Body weights of male mice were unaffected at all time points. *Capsaicin vs. vehicle; $p < 0.05$.

nification (Nikon Eclipse TE2000-E, Tokyo, Japan). Dynamic histomorphometric analysis was performed using commercial software (Bioquant, Nashville, TN) and the results were averaged for the replicate slides from each bone. Mineral apposition rate (MAR), percent mineralizing surface (MS/BS), and bone formation rate (BFR/BS) were quantified for the endosteal and periosteal surfaces.

Serum biomarkers

Blood was collected from capsaicin- and vehicle-treated mice immediately prior to sacrifice for quantification of systemic biomarkers of bone metabolism. Mice were anesthetized with isoflurane and approximately 100–200 μ L of blood was collected retro-orbitally. Samples were allowed to clot for 2–4 hours in an ice bath and then centrifuged at 1000 g for 5 minutes. The supernatants were collected and frozen rapidly to -80°C until analyzed. Serum was analyzed in duplicate to determine the concentrations of carboxy-terminal collagen crosslinks I (CTX-I) and procollagen type 1 amino-terminal propeptide (PINP) using commercial mouse-specific ELISAs

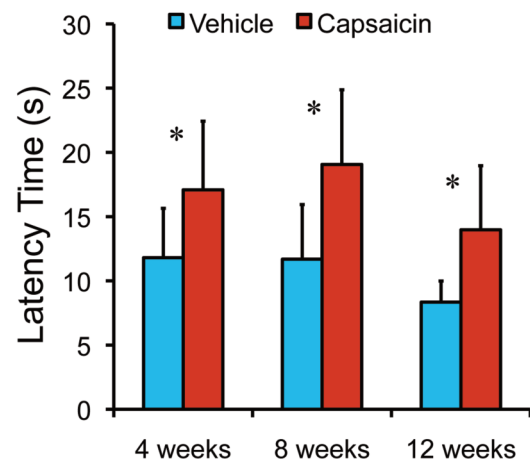


Figure 2. Hot-plate analgesia testing of treated and untreated mice was performed to verify decreased peripheral sensory nerve function. Treatment and age were main effects for latency time ($p < 0.0001$, $p = 0.0052$). Capsaicin-treated mice had significantly longer latency times than vehicle-treated mice at all time points when exposed to a constant 55°C thermal stimulus. *Capsaicin vs. vehicle; $p < 0.05$.

(Cusabio, Wuhan, China) per the manufacturer's instructions. CTX-I is a common biomarker for bone resorption, while PINP is a biomarker for bone formation³⁷.

Statistics

Hot-plate, μ CT, three-point bending, histomorphometry and biomarker data were analyzed using three-way ANOVA stratified by age, sex, and treatment (JMP, SAS Institute Inc., Cary, NC). Between-group differences were analyzed using an unpaired Student's t-test of capsaicin- vs. vehicle-treated mice at each time point. Data are reported as mean \pm SD. Significance was defined as $p < 0.05$.

Results

Mouse body weights

Neonatal capsaicin treatment did not have a statistically significant effect on body weights of male mice; there was no difference in body weight between capsaicin- and vehicle-treated male mice for any time point from weaning through 12 weeks (Figure 1). However, capsaicin treatment significantly affected body weights of female mice at early time points. Female mice treated with capsaicin had 8–13% lower body weights than vehicle-treated female mice from weaning until 47 days of age (Figure 1; $p < 0.05$ for both time points). There were no significant differences in body weights for capsaicin- and vehicle-treated female mice from 8 to 12 weeks of age.

Hot-plate analgesia testing

Mice treated with capsaicin had significantly longer latency times when exposed to the constant thermal stimulus than vehicle-treated mice at all time points examined (Figure 2;

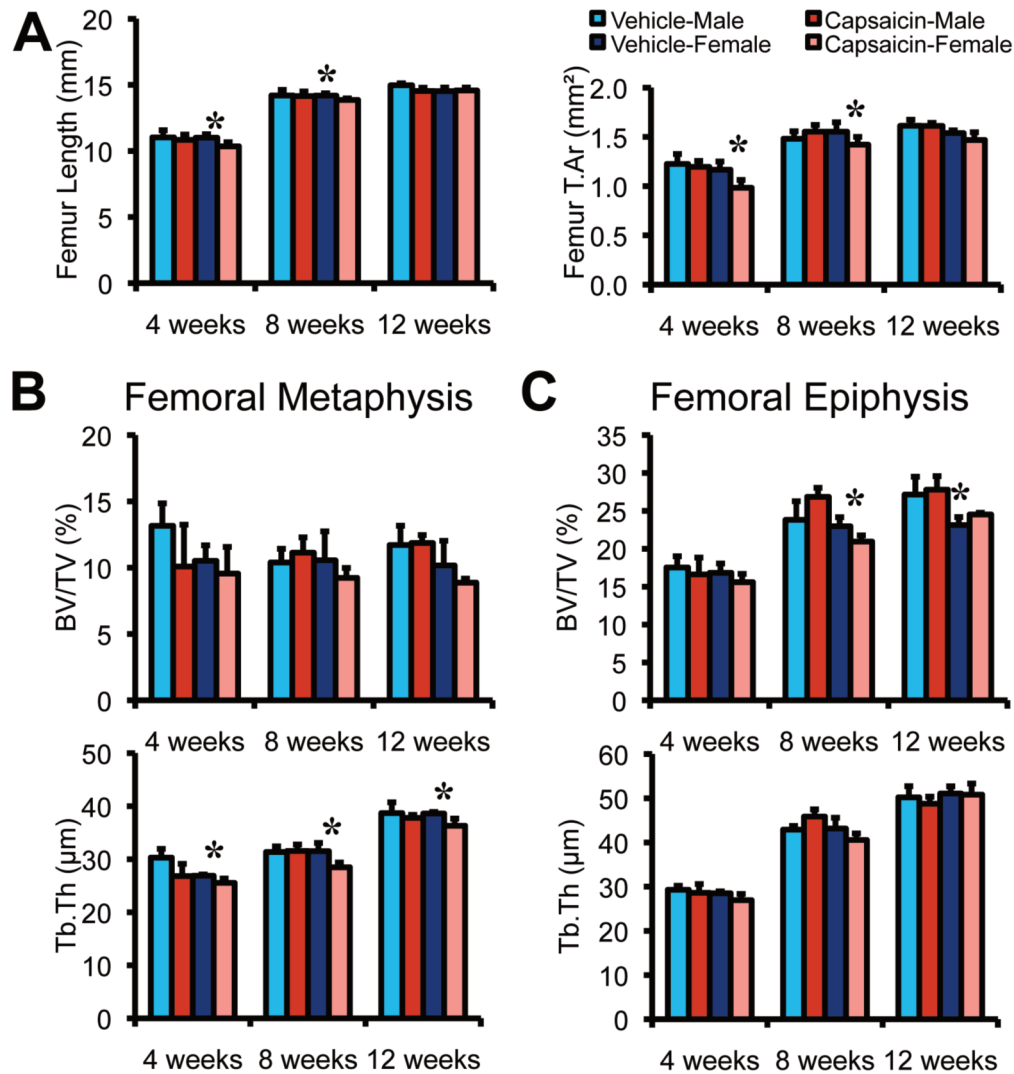


Figure 3. Micro-computed tomography of femoral cortical and trabecular bone. (A) Femur length and total cross-sectional area (Tt.Ar) of the femoral diaphysis were significantly affected in capsaicin-treated female mice. (B) Capsaicin treatment did not significantly affect trabecular bone volume fraction (BV/TV) at the femoral metaphysis but reduced trabecular thickness (Tb.Th) at this location in female mice. (C) Capsaicin treatment significantly affected BV/TV at the femoral epiphysis in female mice, but did not alter Tb.Th at this location. *Capsaicin vs. vehicle; $p < 0.05$.

$p < 0.0001$). For example, at 8 weeks of age, the average latency time of capsaicin-treated mice was 63% longer than that of vehicle-treated mice ($p = 0.0002$), while at 12 weeks of age the latency time was 67% longer for capsaicin-treated mice ($p = 0.0007$). This confirms that mice treated with capsaicin had decreased peripheral sensory nerve function, which persisted until at least 12 weeks of age. Age was also a main effect for latency time ($p = 0.0052$); latency times for both capsaicin- and vehicle-treated mice at 12 weeks of age were significantly shorter than at 4 or 8 weeks.

Micro-computed tomography

Capsaicin treatment resulted in small but significant decreases in bone structure parameters in trabecular bone of mice

relative to vehicle-treated controls (Figure 3). For example, we observed a significant main effect of capsaicin treatment on Tb.Th at the femoral metaphysis and L5 vertebral body ($p = 0.0002$ and 0.0024 , respectively). Female capsaicin-treated mice had 5.2–9.5% lower Tb.Th than vehicle-treated female mice at these sites at each time point ($p = 0.0056$ – 0.049). However, we did not observe a significant effect of capsaicin treatment on BV/TV or Tb.Sp at the femoral metaphysis. At the femoral epiphysis, we observed a significant treatment*sex interaction for Tb.Th. ($p = 0.026$), with female mice being more severely affected by capsaicin treatment than male mice. We also found a significant treatment*sex*age interaction for BV/TV at the femoral epiphysis. For example, female capsaicin-treated mice had 8.7% lower BV/TV at the femoral epi-

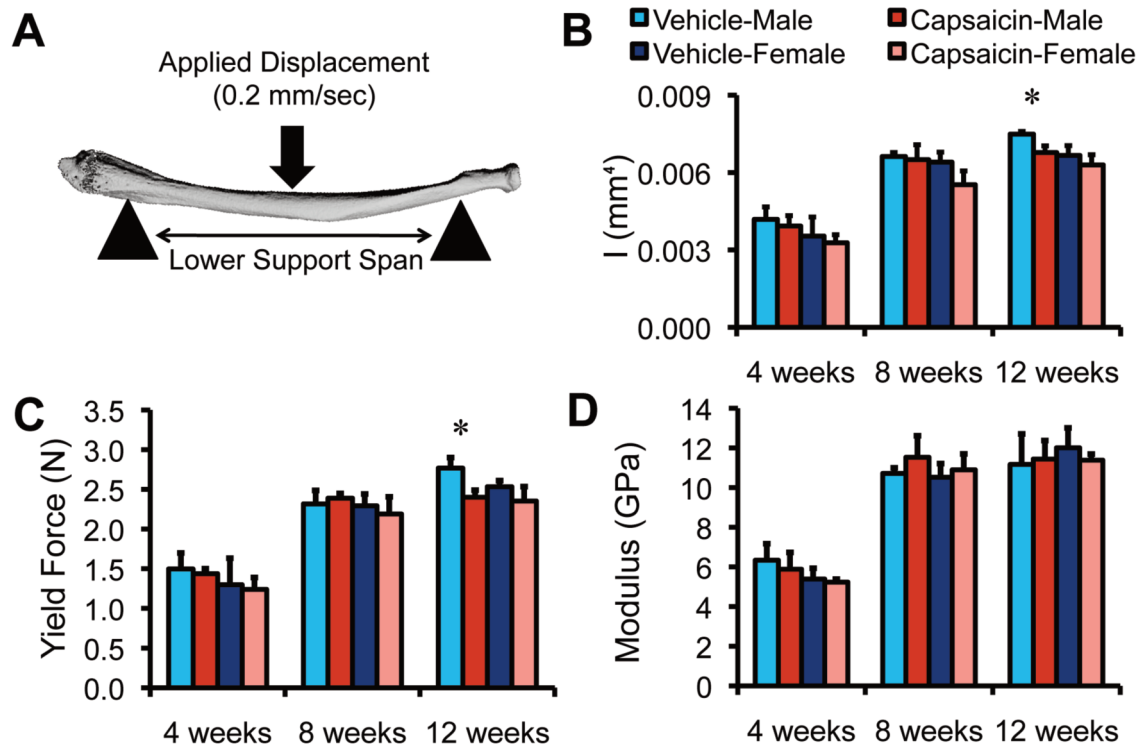


Figure 4. Mechanical properties of radii determined using three-point bending. A micro-computed tomography reconstruction of a representative radius (A) showing the mechanical testing setup. At 12 weeks of age, the moment of inertia (I) (B) and yield force (C) were significantly lower in capsaicin-treated male mice. However, modulus of elasticity (D) was not significantly different between treated and untreated mice. Age was a main effect for I, yield force and modulus. *Capsaicin vs. vehicle; $p < 0.05$.

physis than vehicle-treated female mice at 8 weeks ($p = 0.015$), whereas male mice had similar BV/TV at this time point.

We also observed significant effects of capsaicin treatment on cortical bone structure, with significant main effects of capsaicin treatment on femur length, M.Ar, and Tt.Ar ($p = 0.0094$, 0.026 , and 0.026 , respectively). For example, femurs from female mice treated with capsaicin were 6.0% and 2.3% shorter than femurs from female vehicle-treated mice at 4 and 8 weeks, respectively ($p = 0.034$, 0.0097). At 4 weeks, femurs from female capsaicin-treated mice had 15% smaller M.Ar and 16% smaller Tt.Ar compared with vehicle-treated mice ($p = 0.042$, 0.031). Capsaicin treatment also affected cortical bone of the tibia. There were significant treatment*sex interactions for tibia M.Ar and Tt.Ar ($p = 0.025$, 0.0032). At 4 weeks, the tibias from female capsaicin-treated mice had 16% smaller M.Ar and 15% smaller Tt.Ar compared with vehicle-treated mice ($p = 0.022$, 0.029). We did not observe any significant effects of capsaicin treatment on bone mineral density of either trabecular or cortical bone. As expected, sex and age had significant effects for several measured bone structure parameters.

Three-point bending

Capsaicin treatment significantly decreased the mechanical properties of radii tested with three-point bending. For example, we observed significant main effects of capsaicin treatment on

yield force and moment of inertia ($p = 0.047$, 0.0041). At 12 weeks, radii from male treated mice yielded at 13.3% lower force compared with vehicle-treated male mice ($p = 0.022$). Radii of male mice treated with capsaicin had 9.5% lower moment of inertia than vehicle-treated male mice at 12 weeks ($p = 0.028$). However, calculations of yield stress and modulus of elasticity revealed no significant differences between capsaicin- and vehicle-treated mice (Figure 4). Age was a main effect for all of the mechanical properties measured, while sex was a main effect for yield force and moment of inertia.

Dynamic histomorphometry

Capsaicin treatment significantly altered bone formation parameters in the tibiae of treated mice (Figure 5). We observed significant treatment*sex*age interactions for MAR and BFR at the endosteal surface ($p = 0.023$, 0.020) and for MAR and MS/BS at the periosteal surface ($p = 0.035$, 0.042). For example, at 4 weeks, female capsaicin-treated mice had 39% higher MAR than female vehicle-treated mice at the endosteal surface of the tibia. At 12 weeks, female mice treated with capsaicin had 23.4% lower MS/BS ($p = 0.012$) and 22.4% lower BFR ($p = 0.061$) at the periosteal surface than female vehicle-treated mice. Age was a significant main effect for MAR, MS/BS and BFR at the endosteal and periosteal surfaces.

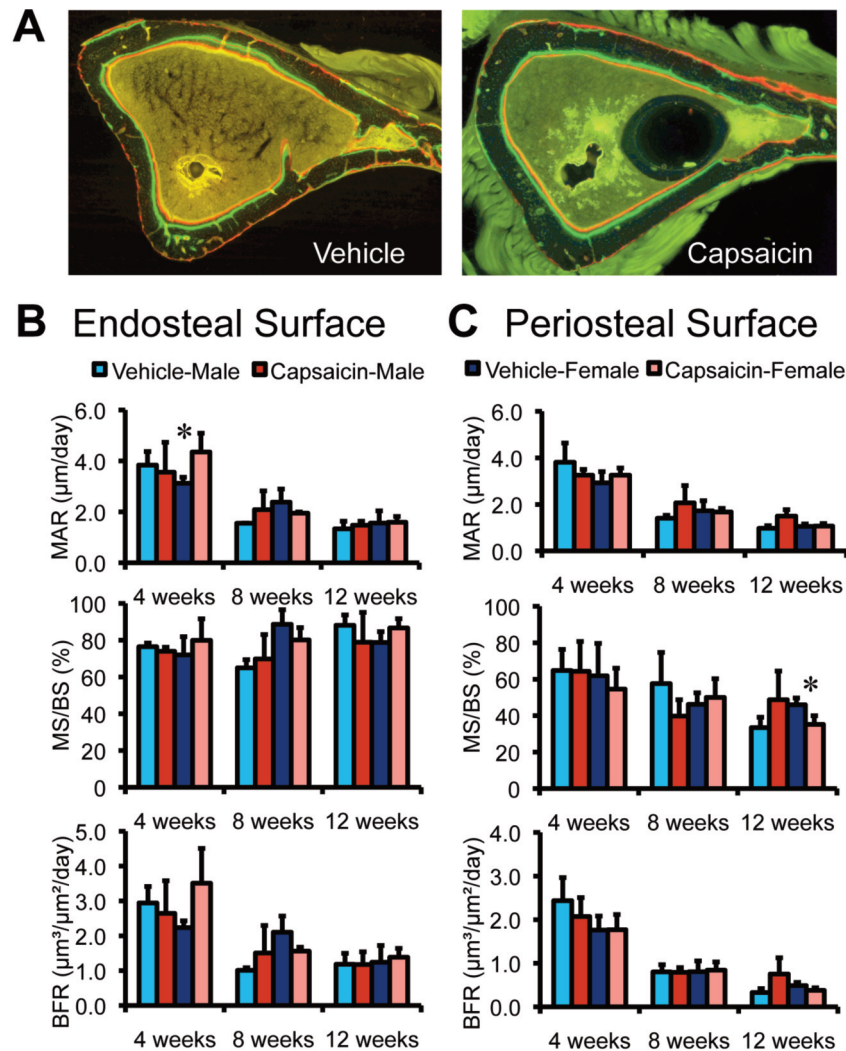


Figure 5. Dynamic histomorphometry was used to identify changes in bone formation rates in capsaicin- and vehicle-treated mice. Fluorescent images (A) show cortical bone from the tibiae of 8 week old female vehicle- and capsaicin-treated mice. Mineral apposition rate (MAR), percent mineralizing surface (MS/BS), and bone formation rate (BFR/BS) were quantified for the endosteal (B) and periosteal surfaces (C). Age was a significant main effect for MAR, MS/BS and BFR at the endosteal and periosteal surfaces. *Capsaicin vs. vehicle; $p < 0.05$.

Serum biomarkers

We did not detect any significant main effects of capsaicin treatment on serum concentrations of CTX-I or P1NP (Figure 6), although male capsaicin-treated mice at 8 weeks of age had 37% lower CTX-I concentrations than vehicle-treated male mice of the same age ($p = 0.046$). We also observed a significant effect of sex on concentrations of CTX-I ($p = 0.043$), with female mice exhibiting lower concentrations of CTX-I than male mice. P1NP serum concentrations did not vary significantly by treatment, sex, or age.

Discussion

In this study, we investigated the role of peripheral sensory nerves in bone metabolism during development in mice. We

found that neonatal capsaicin treatment in mice led to modest and significant decreases in bone structure parameters, but this effect was inconsistent for mechanical properties and bone turnover rate. Capsaicin treatment had the greatest impact on trabecular bone, with treated mice exhibiting lower trabecular thickness at the femoral metaphysis and L5 vertebral body. However, contrary to our initial hypothesis, mice treated with capsaicin did not exhibit reduced mechanical properties or resistance to fracture. Yield stress and elastic modulus were similar between treated and untreated mice, suggesting that gross geometrical changes in the radii caused the lower yield force of capsaicin-treated mice. Also in opposition to our hypothesis, capsaicin treatment did not lead to substantial reductions in bone turnover rate, measured by dynamic histomorphometry and serum concentrations of CTX-I and P1NP. Altogether,

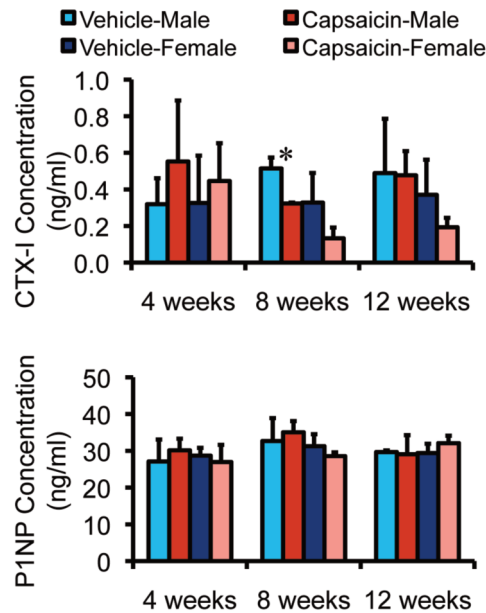


Figure 6. Capsaicin treatment did not significantly affect serum concentrations of CTX-I or P1NP, which were measured using ELISAs. Sex was a main effect for serum concentrations of CTX-I, while P1NP concentrations did not vary significantly by sex or age. *Capsaicin vs. vehicle; $p < 0.05$.

these data indicate that decreased activity of peripheral sensory nerves has a small effect on the trabecular and cortical bone of mice, but that these changes do not result in a meaningful reduction in mechanical properties of whole bones.

In this study we observed only modest differences in bone parameters in capsaicin-treated mice, despite a considerable and sustained decrease in sensory nerve activity. Physiological adaptations during development may allow mice to compensate for the loss of peripheral nerve function, leading to similar development between treated and untreated mice at 12 weeks of age. It is possible that while bone structure and basal metabolic rates are conserved in capsaicin-treated mice as adults, adaptation of bone in response to mechanical loading or injury may be altered, as observed in other mouse models. For example, in a study of adult female mice lacking estrogen receptor- α , the ulnae were only 4% shorter yet demonstrated a three-fold lower osteogenic response to mechanical loading than the ulnae of wild-type littermates³⁸. This is further supported by a previous study using neonatal capsaicin treatment in rats, in which capsaicin treatment did not alter normal bone growth and maintenance, but decreased the local bone remodeling response following molar extraction²². Future studies using this model will investigate the bone adaptation of capsaicin-treated mice to conditions such as increased mechanical loading or injury.

The effect of neonatal capsaicin treatment on bone properties had a differential effect on female mice versus male mice, although the power of this study was not sufficient to fully de-

termine sex-based differences. At 4 and 8 weeks, female capsaicin-treated mice demonstrated the largest differences in bone structure, with shorter femurs with smaller cross-sectional area and trabecular thickness. Male capsaicin-treated mice had significantly lower trabecular thickness at the L5 vertebral body for the first time at 12 weeks of age, and lower moment of inertia and yield force of the radii compared to vehicle-treated mice at this time point. These potential sex-based differences may be related to hormonal fluctuations, as mice reach sexual maturity at 8 weeks of age.

The mouse model used in this study overcomes critical limitations of previous models investigating decreased peripheral nerve function in bone. Capsaicin treatment isolates the effects of decreased sensory nerve function without requiring surgical procedures or impairing motor function. Other studies utilizing capsaicin treatment in adult rats found that capsaicin-sensitive neurons contribute to the maintenance of trabecular bone^{39,40}. However, capsaicin directly interacts with bone cells^{41,42}, making it difficult to separate the primary effect on bone cells from the secondary effect of decreased peripheral nerve function, as both osteoclasts and osteoblasts express receptors for capsaicin^{43,44}. Treating mice as neonates instead of at maturity allows time for recovery of normal bone metabolism, and therefore isolates the effects of sensory nerve inactivation without the complication of direct action on bone cells inherent in adult treatment.

In this study we were able to detect small but significant differences in trabecular and cortical bone structure, but were unable to detect differences in bone mineral density or bone mechanical properties. This suggests that peripheral nerve inactivation may influence bone quantity, but not bone quality. If this is the case, differences in bone formation or resorption would be expected. However, imprecise methods for detecting bone turnover rates may have limited our observation of changes in capsaicin-treated mice. We were able to detect small changes in bone structure because of the high precision of micro-computed tomography. However, we were unable to detect small differences using lower resolution methods such as dynamic histomorphometry and quantification of serum biomarkers. Both of these techniques were further limited by scope: our histomorphometric analysis was limited to cortical bone, and biomarker concentrations were measured from the serum of developing mice. Similarly, three-point bending of radii may not have had adequate sensitivity to fully capture changes in bone mechanical properties.

This study is limited because it did not directly investigate the effects of capsaicin treatment on sensory neurons in bone. While we verified decreased peripheral sensory nerve function using response to a thermal stimulus, we did not perform a direct quantification in bone, such as counting neurons remaining after treatment or quantification of neuropeptides in bone. It is well established that capsaicin treatment does not affect motor neurons, however it is possible that the reduction in peripheral sensory nerve function could alter spontaneous motor activity in mice. Quantification of activity levels in vehicle- and capsaicin-treated mice could further establish that differ-

ences in bone structure are due to decreased sensory nerve function rather than decreased mechanical loading of bone. This study also did not investigate the response of bone to differing levels of sensory nerve inactivation. A more thorough disruption of peripheral sensory nerve function could have a more dramatic effect on bone metabolism than was observed in this study. Further, in this study we used neonatal capsaicin treatment as a surrogate for age-related degeneration of peripheral nerves, but did not directly make comparisons with aged animals. It is possible that aged mice demonstrate a different pattern of neural degeneration than mice treated with capsaicin as neonates. However, we were able to study the isolated effects of decreased peripheral sensory nerve function in mice up to skeletal maturity.

This study further supports a role for peripheral sensory nerves in bone metabolism. Using neonatal capsaicin treatment, we established a mouse model of decreased peripheral sensory nerve function and observed resulting changes in bone structure. We observed only small changes in bone structure with sensory nerve inactivation, with no notable changes in bone metabolism or bone quality. Therefore age-related changes in sensory innervation may not be clinically meaningful with respect to bone strength under normal loading conditions. It is possible that capsaicin-sensitive neurons may become important for bone under stress conditions such as increased mechanical loading or injury. Future studies will investigate this potential role of peripheral sensory nerves in bone adaptation.

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